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ORIGINAL ARTICLE

Guido F. Laube · Vanita Shah · Victoria C. Stewart · Iain P. Hargreaves · Mushfequr R. Haq · Simon J. R. Heales · William G. van't Hoff

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Abstract We have determined levels of glutathione (GSH), ATP, mitochondrial complex activity and apoptosis rate in proximal tubular cells (PTCs) exfoliated from urine in cystinotic (n=9) and control (n=9) children. Intracellular GSH was significantly depleted in cystinotic PTCs compared with controls (6.8 nmol GSH/mg protein vs 11.8 nmol GSH/mg protein; P<0.001), but there were no significant differences in mitochondrial complex activities or ATP levels under basal conditions. Cystinotic PTCs showed significantly increased apoptosis rate. After PTCs had been stressed by hypoxia, there was further depletion of GSH in cystinotic and control PTCs (2.4 nmol GSH/mg protein vs 7.2 nmol GSH/mg protein; P<0.001). Hypoxic stress led to increased complex I and complex IV activities in control but not in cystinotic PTCs. ATP levels were significantly reduced in cystinotic PTCs after hypoxic stress (12.2 nmol/mg protein vs 26.9 nmol/mg protein; P<0.001). GSH depletion occurs in this in vitro model of cystinotic PTCs, is exaggerated by hypoxic stress and may contribute to reduced ATP and failure to increase complex I/IV activities. Apoptotic rate is also increased, and these mechanisms may contribute to cellular dysfunction in cultured, human cystinotic PTCs.

Keywords Cystinosis · Renal Fanconi syndrome · Glutathione · Apoptosis · Mitochondria

G. F. Laube · V. Shah · M. R. Haq · W. G. van't Hoff
Nephro-urology Unit, Institute of Child Health,
University College London Medical School,
London, UK

V. C. Stewart · I. P. Hargreaves · S. J. R. Heales
Department of Molecular Neurosciences,
Institute of Neurology, University College Medical School,
London, UK

I. P. Hargreaves I · S. J. R. Heales
Neurometabolic Unit,
National Hospital for Neurology and Neurosurgery,
Queen Square,
London, UK

G. F. Laube (✉)
University Children's Hospital,
Steinwiesstrasse 75,
8032 Zurich, Switzerland
e-mail: guido.laube@kispi.unizh.ch
Tel.: +41-44-2667390
Fax: +41-44-2667861

Introduction

Cystinosis is a rare, autosomal, recessive disorder characterised by defective lysosomal transport of cystine, leading to intra-lysosomal cystine accumulation [1–7]. Infantile nephropathic cystinosis is characterised by severe renal proximal tubular dysfunction [renal Fanconi syndrome (FS)], but the link between the intra-lysosomal cystine accumulation and the FS is still unclear. Normal rabbit and rat proximal tubules loaded with cystine by incubation with cystine dimethyl ester (CDME) have intracellular cystine concentrations similar to those of cystinotic cells and exhibit defective tubular transport as in the FS [8–10]. CDME-loaded tubules exhibit reduced ATP levels and have impaired oxygen consumption [9, 10], consistent with defective oxidative phosphorylation and/or mitochondrial dysfunction. Defects in mitochondrial function have also been found in animal models of FS, e.g. tyrosinaemia, and genetic mitochondrial disorders can lead to a profound FS [11, 12]. Cystine has also been found to directly inhibit Na^+-dependent co-transporters in cystine-loaded proximal tubule cells [13]. Recent work has indicated that cultured fibroblasts from cystinotic patients exhibit an increased apoptotic rate compared with those from healthy subjects and that this can be negated by pre-treatment with cysteamine [14].

The tri-peptide glutathione (GSH) has a major role in cellular redox reactions and thiol-ether formation. Under hypoxic stress, GSH is oxidised to GSH-disulphide, defining a rupture between pro-oxidant and anti-oxidant systems. The existence of such oxidative stress is established in patients with chronic renal disease [15]. Furthermore, the de novo synthesis of the key cellular

antioxidant, GSH, may be dependent on cytosolic cystine/cysteine availability [16].

In order to study proximal tubular cell (PTC) dysfunction in cystinosis we used an in vitro model by culturing and characterising PTCs from cystinotic patients and controls [17]. We hypothesised that mitochondrial dysfunction and increased apoptosis rate might contribute to the FS in cystinosis. We also postulated that GSH status might be compromised in cystinotic cells. After growing and characterising PTCs from patients and controls, we studied mitochondrial function, GSH status and the rate of apoptosis.

Methods

Cell culture and characterisation

PTCs were collected from nine early-onset cystinosis patients (prior to end stage renal failure; mean creatinine 86 μmol/l) and nine children in a control group, consisting of patients from the cardiac intensive care unit, 2 days after surgery [17]. These "control" children were selected as they routinely had urethral catheters but were haemodynamically stable and had no clinical or biochemical evidence of renal impairment at the time of study. All the cystinosis patients had been on long-term treatment with cysteamine 40–60 mg/kg per day, with trough leucocyte cystine concentrations maintained generally at <1 nmol ½ cystine/mg protein. All participating families provided informed written consent, and the study received ethical approval. Cells were frozen in liquid nitrogen at various stages of culture prior to experiments. Cells exfoliated in the children's urine were cultured and characterised as predominantly PTCs as previously described [17, 18]. Cells were grown under basal (21%) or hypoxic conditions (3% oxygen for 48 h, a value previously shown to elucidate a stress response [19]). Cells used in the studies were taken from confluent monolayers in cell passages 1–3. PTCs from individual patients were treated as separate isolates, and each experiment was repeated in triplicate.

Measurement of intracellular ATP and glutathione

PTCs were grown in 25 cm^2 flasks until confluent and then washed twice with phosphate-buffered saline PBS solution. Perchloric acid (0.4 M) (Aristar, BDH Laboratory Supplies England) was added to the flask [to destroy the cell membrane, as the supernatant fraction contains the intracellular content (including ATP)], which was left on ice for 15 min. After being transferred to an Eppendorf tube, the sample was centrifuged and the supernatant decanted. The pH was adjusted to a value of 6.0–7.0 with potassium hydroxide (KOH, 3 M) (Sigma, St. Louis, USA). After centrifugation, the supernatant was transferred to a new Eppendorf tube ready for the ATP determination, measured by high-performance liquid chromatography [20]. For determination of GSH, PTCs were grown in 25 cm^2 flasks until confluent and again scraped in PBS. After centrifugation the supernatant was aspirated and the cell pellet resuspended in PBS before being frozen at −70°C degrees prior measurement of total GSH [21]. Spiking of samples with 2.5 μM GSH revealed a 98±2% (n=6) recovery of GSH. Furthermore, the GSH concentration in the acid extracts was stable for 24 h at room temperature and for at least 1 year at −70°C [22]).

Enzyme activity determination (measurement of mitochondrial complexes I to IV)

PTCs were grown in 75 cm^2 flasks until confluent and then harvested, pelleted and resuspended in 0.25 M sucrose solution containing 2 mM HEPES, pH 7.2 (Sigma). After three cycles of freeze-thawing to lyse membranes, mitochondrial enzymes [NADH CoQ_1 reductase (complex I; EC 1.6.5.3); succinate dehydrogenase (complex II; EC 1.3.5.1); ubiquinol-cytochrome c reductase (complex III; EC 1.10.2.2); cytochrome c oxidase (complex IV; EC 1.9.3.1); citrate synthase (EC 4.1.3.7)] were assayed spectrophotometrically by established methods [23–26]. Enzyme activities were expressed as nanomoles per minute per milligramme of protein, except that of cytochrome c oxidase, which was expressed as the first-order rate constant k per minute per milligramme of protein. The activity of citrate synthase (CS) was used as a marker of mitochondrial enrichment.

Measurement of apoptosis rate

Induction and measurements of apoptosis were performed with commercially available reagents. PTCs were grown in 25 cm^2 flasks until confluent and then exposed to apoptotic triggers: TNF-α (2 ng/ml; Cell Sciences, USA) with actinomycin-D (2.5 μg/ml; Sigma) and anti-Fas antibody (500 ng/ml; TNB Laboratories, Canada) for 16 h. After exposure, the PTCs were harvested and caspase-3 activity was measured with the caspase-3 colorimetric activity assay kit [CN Bioscience (UK)]. We then used the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method, which utilises terminal deoxynucleotidyl transferase to label the ends of double-stranded DNA breaks, which occur in apoptotic cells, with fluorescein isothiocyanate (FITC)-conjugated dUTP. PTCs were grown, fixed and permeabilised with methanol/acetone (1:1) for 5 min on ice and stained using the TUNEL enzymatic labelling assay (Roche Diagnostics, Mannheim, Germany). We counted the cells by fluorescence microscopy, scoring at least three view fields for fluorescence in triplicate after counting the same field

by light microscopy. The apoptosis rate is given as a percentage and results from the total number of PTCs that fluoresce divided by the total PTCs in the field.

Statistics

Descriptive data are presented as geometric means and ranges and compared using unpaired Student's two-tailed *t*-tests.

Results

Cells became confluent after 2–4 weeks, were characterised as predominantly PTC phenotype and accumulated cystine as previously demonstrated [17, 18]. Cells were studied between passages 1 and 3, and there was no significant effect of passage number on any of the results presented below (data not shown).

Basal conditions (21% oxygen)

The results are summarised in Figs. 1 and 2 (data relative to citrate synthase to correct for mitochondrial enrichment). Individual variation among cystinotic and control patient samples was small and statistically insignificant. GSH levels were significantly lower in cystinotic PTCs than in controls (6.8±2.8 nmol GSH/mg protein vs 11.8±2.8 nmol GSH/mg protein; $P<0.001$; Fig. 1a). There were no significant differences in ATP levels (Fig. 1b) or in mitochondrial complex activities in cystinotic PTCs compared to controls under basal conditions (Fig. 2).

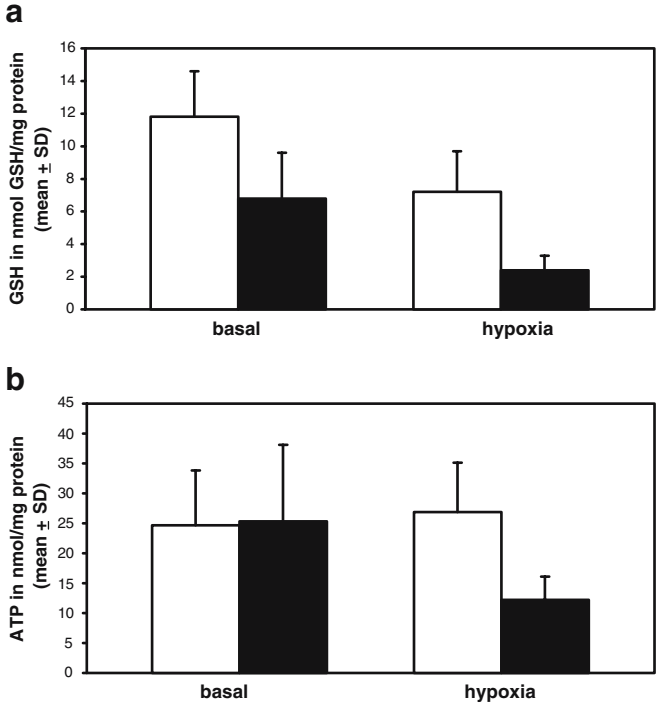

Fig. 1 Glutathione (**a**) in nanomoles/mg protein and ATP (**b**) in nanomoles/mg protein in cystinotic PTCs (*black*) and control PTCs (*white*) (mean ± SD) after PTCs were grown under basal conditions (21% oxygen) and under hypoxic conditions (3% oxygen). Statistical comparison (Student's two-tailed *t*-test) between patients and controls for GSH (basal/hypoxia) and ATP (hypoxia): $P<0.001$

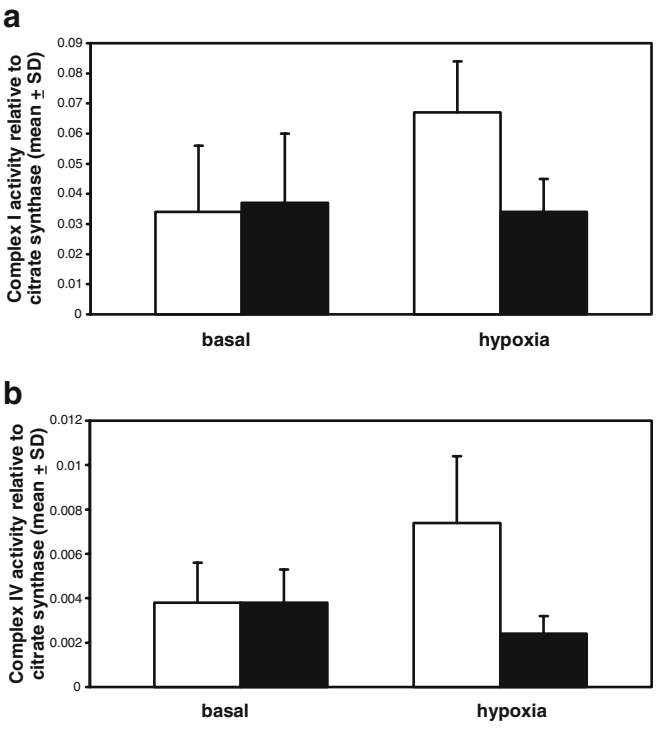

Fig. 2 Mitochondrial function in cystinotic PTCs (*black*) and control PTCs (*white*) (mean ± SD) after PTCs were grown under basal conditions (21% oxygen) and under hypoxic conditions (3% oxygen). **a** Mitochondrial complex I activity related to citrate synthase. **b** Mitochondrial complex IV activity related to citrate synthase. Statistical comparison (Student's two-tailed *t*-test) between patients and controls for mitochondrial complex I/IV activity (hypoxia): $P<0.001$

Hypoxic conditions (3% oxygen)

Hypoxic stress was associated with a further significant deletion in GSH status in the cystinotic PTCs compared with control PTCs (2.4±0.9 nmol GSH/mg protein vs 7.2± 2.5 nmol GSH/mg protein, $P<0.001$; Fig. 1a). Additionally, ATP levels were significantly decreased when compared with those of control PTCs (12.2±3.9 nmol/mg protein vs 26.9±8.2 nmol/mg protein, $P<0.001$; Fig. 1b). In control cells, hypoxia was associated with an increase in complex I (0.067±0.017 vs 0.034±0.011, $P<0.001$; Fig. 2a) and complex IV (0.0074±0.003 vs. 0.0024±0.0008, $P<0.001$; Fig. 2b) activities. However, in the cystinotic PTCs, mitochondrial complexes I and IV activities were not significantly different from those under basal conditions (Fig. 2) (complex I basal activity 0.037±0.023 vs complex I hypoxic activity 0.034±0.011; complex IV basal activity 0.0038±0.0015 vs complex IV hypoxic activity 0.0024± 0.0008). In all cases there were no significant changes in

complexes II and III. The variable effects of hypoxia on complex I and IV activities between cystinotic and control cells compared with the activities of complexes II and III are consistent with a specific effect on complexes I and IV rather than a generalised loss of mitochondrial or whole-cell viability.

Apoptosis rate in cystinotic PTCs

Apoptosis rates, induced by TNF-α or anti-Fas antibody and measured by TUNEL and caspase-3 activity, are summarised in Table 1. The apoptosis rate in cystinotic PTCs, as determined by caspase-3 activity, was significantly ($P<0.001$) increased both with and without anti-Fas antibody trigger. Measurements also showed higher, but not significant, caspase-3 levels in cystinotic PTCs when triggered with TNF-α. Similar results were obtained when PTCs were analysed by TUNEL, and significant ($P<0.001$) differences could be shown using anti-Fas antibody and TNF-α. Immunohistochemistry demonstrated an increased apoptosis rate in cystinotic PTCs compared with control PTCs (data not shown).

Discussion

The pathogenesis of proximal tubular dysfunction in cystinosis is not fully understood. Generalised proximal tubular dysfunction (FS) can occur in a heterogeneous group of genetic disorders and acquired diseases and can also be caused by drugs or toxins. A variety of pathogenetic mechanisms has been described (reviewed in [27]). There is increasing evidence that defects in the endocytic pathway are implicated in the abnormal urinary losses of low molecular weight proteins in FS (e.g. in Dent's disease and in Lowe's syndrome [28]). Abnormalities in mitochondrial function or in sodium-coupled co-transport have been suggested as mediators of the abnormal electrolyte losses. We suggest that some of these mechanisms may co-exist in the various disorders leading to the renal FS.

In cystinosis, data from an animal model showed impaired mitochondrial function and oxidative phosphorylation to be responsible for the FS [8–10]. In addition, human cystinotic fibroblasts show increased apoptosis rate, suggesting that this may also occur in PTCs [14]. Electrophysiological studies have shown inhibition of Na^+-dependent transporters in cystine-loaded human renal cells [13]. However, these studies have been undertaken in cells loaded with CDME in which cystinosin function was *normal* and, therefore, may not be relevant to native cystinotic cells in which the function of cystinosin is defective. A mouse knock-out model of cystinosis exhibits cystine accumulation but no tubulopathy, limiting its potential as a model in which to study the pathogenesis of FS [29]. We therefore undertook studies in human PTCs cultured from the urine of cystinosis children and controls [17]. Cystine levels in the cystinotic PTCs were significantly elevated compared with the control PTCs and similar to those found in leucocytes from cystinotic patients [17, 18].

We observed apparent loss of GSH status in cystinotic PTCs under basal conditions; however, mitochondrial complex activities and ATP levels were unchanged. Hypoxic stress led to a decrease in GSH status in control cells and a further decrease in cystinotic cells. Our previous experience with other cell types frozen at −70°C has not identified any apparent effects of storage time on GSH status. However, in this series of experiments utilising cystinotic cells, it is possible that high disulphide exchange secondary to the high cystine disulphide pool may have contributed to loss of GSH status. It is possible that this might explain some of the differences observed between the cystinotic and control cell results. Previous data on GSH levels in cystinosis fibroblasts have shown a decrease, in one study [30], but no statistical difference from controls in two other studies [31, 32], although in the latter, stress led to significant GSH depletion in cystinotic cells [32]. It is possible that these differences relate to our use of PTCs (a cell type primarily affected in cystinosis) rather than fibroblasts. It is known that discrepancies can occur between studies undertaken in fibroblasts and those undertaken in target organ tissues (e.g. muscle in mitochondrial disorders [33]).

An increase in urinary 5-oxoproline (pyroglutamate) has previously been demonstrated in cystinotic patients [34], and it has been speculated that this might arise from perturbation of the γ-glutamyl cycle, which could be associated with GSH depletion. Cystinotic fibroblasts exhibited raised pyroglutamate levels, compared with controls, and the difference became more marked after cells were stressed [32]. However, known inherited defects of the γ-glutamyl cycle are not known to be associated with Fanconi syndrome, so it is not yet clear how these previous observations are related to the pathogenesis of the tubular dysfunction.

Table 1 Apoptosis rate in cystinotic PTCs and in control PTCs (mean ± SD) measured by caspase-3 activity (in picomoles/minute per milligramme) and TUNEL (as a percentage)

Parameter		No trigger	TNF-α	Anti-Fas antibody
Caspase-3 activity	Patients	$^{(1)}$90.8±21.7	66.1±14.4	$^{(4)}$85.7±17.4
	Controls	$^{(1)}$49±15.2	52.2±22.2	$^{(4)}$47.4±16.8
TUNEL	Patients	$^{(2)}$15.5±3.1	$^{(3)}$18.1±5.3	$^{(5)}$20.5±5.2
	Controls	$^{(2)}$7.3±1.5	$^{(3)}$9.3±1.9	$^{(5)}$10.5±3.2

$^{(1–5)}$Statistical comparison (Student's two-tailed *t*-test) between patients and controls: $P<0.001$

GSH biosynthesis is likely to be dependent on a cytosolic supply of cysteine/cystine [15, 16]. Although intracellular cystine is elevated in cystinotic cells, it is compartmentalised within lysosomes secondary to defective/absent cystinosin function, and previous work on cystinotic fibroblasts has indicated normal cytosolic levels of cystine and cysteine [35]. The exact mechanism whereby GSH status could be compromised in cystinotic PTCs is not yet known. Further evidence of the role of GSH depletion in the pathogenesis of proximal tubular transport would come from studies of the expression and function of co-transporters in this model of cystinotic PTCs and the effect of supplementing GSH in this system.

GSH has a number of key cellular roles, including post-translational protein modification and xenobiotic detoxification, and it acts as a major antioxidant. Deficiency of GSH has also been implicated in other forms of the renal FS that occurs after ifosfamide therapy or in Balkan endemic nephropathy (BEN), in which ochratoxin A is considered to be the environmental toxin [36]. Ifosfamide toxicity may be mediated by its interaction with γ-glutamyl transpeptidase and by hepatic metabolism to chloroacetaldehyde [37]. Incubation of chloroacetaldehyde with isolated human renal proximal tubules was associated with cellular depletion of GSH, coenzyme A, acetyl-coenzyme A and ATP [38]. Wistar rats injected with ifosfamide develop renal FS, associated with GSH depletion [38]. Concurrent treatment with melatonin attenuates the GSH depletion and attenuates the renal FS [38]. Ochratoxin A exposure to rat proximal tubular cells led to elevation of reactive oxygen species and depletion of cellular GSH [39]. Ochratoxin A also increases caspase-3 and induces apoptosis in human proximal tubular cells [39]. Moreover, augmenting intracellular GSH may have a protective effect. Rat proximal tubular cells, transfected to over express the dicarboxylate carrier (thereby increasing mitochondrial GSH), exhibited less apoptosis after exposure to tert-butyl hydroperoxide or S-(1,2-dichlorovinyl)-L-cysteine than did wild-type cells [40].

Depletion of GSH is also associated with the initiation of apoptosis [41]. In support of this, we also observed increased an apoptosis rate in cystinotic PTCs after triggering the cells with TNF-α and anti-Fas. Triggering the PTCs with TNF-α was not as effective as with anti-Fas antibody or even without the use of any trigger, but, as shown in endothelial cells, TNF-α may not be a successful trigger in all types of cells [42].

It is increasingly recognised that apoptosis is linked to intracellular biochemical events. In particular, mitochondria have a key role in cell death [42–46]. Firstly cells accumulate effector molecules directly acting on mitochondria and causing mitochondrial membrane permeabilisation. This is followed by decision, the effective act of mitochondrial membrane permeabilisation and degradation, the triggering of a series of catabolic reactions due to the release of factors (such as cytochrome *c*) that induce cell death, either by apoptosis or by necrosis. Park and Thoene first described increased apoptotic rates in cultured cystinotic fibroblasts [14] and speculated that apoptosis may be a key factor in the development of the multisystem phenotype of cystinosis [14]. Our data in proximal tubular cells support their data, but further work is needed to understand the contribution of each of these different pathogenetic mechanisms in cystinosis.

We found no evidence, in cystinotic cells grown under basal conditions, of impaired activity of any of the components of the mitochondrial electron transport chain. However, the assays employed will only detect: (a) irreversible damage that is sufficient to manifest itself under the saturating assay conditions employed or (b) gross changes in the steady state level of a particular enzyme complex. Thus, reversible inhibition of the electron transport chain, e.g. by nitric oxide [47], that may occur within the intact cell, will not be detected. Whilst there was no detectable evidence for an impairment of the mitochondrial electron transport chain, it is entirely possible that other factors related to mitochondrial function are perturbed in cystinotic cells, e.g. ATP synthetase, mitochondrial membrane potential and permeability transition.

In contrast to the control cells, in the cystinotic cells hypoxia was associated with a loss of ATP. Inspection of the activities of the mitochondrial respiratory chain complexes revealed that, in the control cells, hypoxia was associated with increased activities of mitochondrial complexes I and IV. Increases in the specific activities of complexes I and IV have also been demonstrated in other model systems of oxidative stress, including GSH deficiency, neurodegenerative disorders and septic shock [48–51]. These studies suggest that, when molecular oxygen is limiting, there is normally a compensatory increase in complexes I and IV of the electron transport chain in order to maintain the availability of ATP. Our data, however, suggest that in cystinotic cells this protective mechanism may be lost. Sodium-dependent co-transport in the proximal tubule depends on a sodium gradient, maintained by adequate function of basolateral ATP-driven NaK ATPase. Mitochondrial dysfunction with subsequent ATP depletion will secondarily reduce NaK ATPase function. In addition, recent work, in a rat model of ischaemic tubular damage, found that ATP depletion disrupted the ankyrin–spectrin complex that holds NaK ATPase to the basolateral membrane, leading to its mislocation to the cytosol [52]. The net result of reduced NaK ATPase function is a reduced gradient for sodium-coupled co-transport and, hence, increased urinary solute losses.

In conclusion, this study has demonstrated, in human cystinotic proximal tubular cells, depletion of GSH and an increased susceptibility for apoptotic cell death. In addition, our data suggest that, under hypoxic stress, cystinotic cells may have lost the ability to up-regulate key components of the mitochondrial electron transport chain and, hence, preserve cellular ATP. The link between intralysosomal cystine accumulation, secondary to defective cystinosin function, and GSH depletion remains unclear. Whatever the mechanism, we speculate that GSH deficiency and increased apoptosis rate are implicated in the pathogenesis of proximal tubular dysfunction in cystinosis.

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